High-Voltage Thin-Layer Electrophoretic Method for Detecting Histidinemia

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A high-voltage thin-layer electrophoretic method is described for separating urinary histidine and imidazoleacetic, imidazolelactic, and imidazolepyruvic acids. The procedure provides a reliable confirmation of the diagnosis of histidinemia.

Additional Keyphrases imidazoleacetic, -lactic, and -pyruvic acids in urine

Histidinemia, an inborn error of metabolism with an autosomal recessive pattern of inheritance, is characterized by increased urinary excretion of histidine, and of imidazoleacetic, imidazolelactic, and imidazolepyruvic acids. A rapid, effective method for separating and identifying these compounds would clearly facilitate detection and diagnosis of the disorder.

Recently, Berry and Poncet (1) reported separation of histidine, imidazoleacetic acid, and imidazolelactic acid by unidimensional paper chromatography. This method is quite satisfactory for separation of these compounds, but the chromatograms require overnight development. Hummel (5) has also described a method for identifying imidazoles by thin-layer chromatography on cellulose, which is suitable for confirming the diagnosis of histidinemia. However, the procedure involves double chromatography to eliminate the "salt-effect" that results when undesalted urine is applied to the thin-layer plate.

To eliminate some of the difficulties inherent in the paper and thin-layer chromatographic methods, we devised a procedure for high-voltage thin-layer electrophoresis to separate and identify histidine and its imidazole derivatives in urine. The small volume of urine required (1.5 µl to 15 µl per cm streaked) can be quickly applied to the thin-layer plate, urine samples need not be desalted, and the components of interest are separated in 30 min. In addition, the procedure is quite sensitive. We have demonstrated that, in a single sample application to the thin-layer plate, as little as 20 mg of histidine can be detected per liter of urine.

Materials and Methods

Thirty grams of cellulose 300 MN (Mackerey, Nagel & Co., Düren, West Germany) was blended for 30 s with 180 ml of distilled water and was then spread on the glass plates in layers 500 µ thick with a "Unoplan Spreader" (Shandon Scientific, Inc., Sewickley, Pa. 15143). The plates were then air-dried, activated by heating at 110°C for 30 min, and stored under room conditions for later use. It was not necessary to reactiviate the plates just prior to use. A line was lightly drawn across the plate, 5 cm from the bottom edge, and divided into 1.5-cm segments, separated by 1 cm. An appropriate amount of urine or reference compound was applied to each 1.5-cm segment with a mechanical streaker (Applied Science Laboratories, Inc., State College, Pa. 16801).

A reference mixture containing 1 mg/ml each of histidine and imidazoleacetic, imidazolelactic, and imidazolepyruvic acids was made up in formic acid–acetic acid buffer, pH 1.9. Urocanic acid at a 1 mg/ml concentration was also included as a reference compound because of its occasional presence in normal urine specimens (6). An amount of urine corresponding to about 3 µg of creatinine per centimeter of streak was applied to the thin-layer plate. [Urinary creatinine was determined by a modification of the automated procedure of Chasson et al. (6).]
A Model #FP-18 high-voltage electrophoresis apparatus (Savant Instruments Inc., Hicksville, N.Y. 11801) was used for the thin-layer separations. The temperature of the coolant was maintained at −5°C. The pH 1.9 buffer consisted of 59.2 ml of glacial acetic acid and 31.2 ml of formic acid, diluted to 1 liter with distilled water (4). The wicks were double-thicknesses of Whatman No. 1 filter paper, previously soaked in this buffer.

While sample streaks were protected by covering them with a narrow strip of Whatman No. 1 filter paper, thin-layer plates were sprayed with the pH 1.9 buffer until they were uniformly saturated. The buffer from the exposed saturated portions of the plate was then allowed to migrate in from above and below to wet the sample streaks. The plates were placed on the electrophoresis apparatus with the sample streaks toward the cathode end. The filter paper wicks were then placed in position 2 cm from the ends of the plate and allowed to equilibrate for 5 min. One thousand volts (1.5–2.0 mA/cm² of plate width) was applied for 30 min, after which the plates were removed from the apparatus and dried in a current of warm air.

When the plates were thoroughly dry, the spots were made visible by spraying with diazotized sulfanilic acid (Pauly’s reagent). This reagent was freshly prepared in an ice bath by mixing 5 ml of NaNO₂ (50 g/liter) and 5 ml of sulfanilic acid solution (9 g of sulfanilic acid and 90 ml of coned HCl, diluted to 1 liter with distilled water). Immediately before use, 10 ml of Na₂CO₃ solution (100 g/liter) was added to the mixture.

Results and Discussion

The electrophoretic separation of histidine, the imidazole derivatives of histidine, and urocanic acid is shown in Figure 1. Urocanic and imidazolelactic acids did not always separate completely, but their colors after reaction with Pauly’s reagent were different (urocanic acid: brown; imidazolelactic: red), so each could be identified easily. By use of the technique of Humbel (5), urocanic and imidazolelactic acids could be positively identified after chromatographic separation in the second dimension.

High-voltage thin-layer electrophoresis of the urine specimens from three different patients with histidinemia demonstrated the excretion of distinctly increased amounts of histidine, imidazoleacetic acid, and imidazolelactic acid as compared with normal subjects. Figure 1 shows a typical pattern from one of the patients compared to urine from a normal person. Little or no imidazolepyruvic acid was found in the urine of the patients tested with known histidinemia, probably because the urine specimens were not fresh. However, imidazolepyruvic acid can be detected in the urine of patients with the disorder by this method, and it is important to remember that fresh urines should be used if all three imidazole derivatives of histidine are to be demonstrated. Our studies indicate that the high-voltage thin-layer electrophoretic method is rapid, sensitive, and reliable, and should be very useful for detecting or corroborating the diagnosis of histidinemia.

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References